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14. ABSTRACT
In this first year of funding we characterized the direct actions of adiponectin on murine T lymphocytes in vitro. Adiponectin induced lymphocyte proliferation in the absence of the specific antigen against which they were activated in vivo (MOG_{35-55}). We will further study the mechanisms leading to these phenomena in the next year by silencing expression of adiponectin receptors on murine T cells. We have also performed experiments an in vitro model of blood brain barrier (BBB). We demonstrated expression of adiponectin receptors on astrocytes and endothelial cells, the two BBB components. Next, we tested adiponectin effects on the BBB. In vitro barrier integrity was assessed by measurement of transendothelial electrical resistance (TEER) or diffusion of fluorescently labeled solutes (fluorescein-dextran). Treatment with adiponectin significantly increases TEER and decreases the diffusivity of fluorescein-dextran compared to treatment with vehicle (PBS). These findings suggest that adiponectin in vitro decreases BBB permeability. No significant effects of adiponectin were observed on level of expression of the endothelial molecules VCAM and VE-cadherin. Furthermore, we have started studying expression of adiponectin receptors on peripheral blood immune cells in MS patients and controls by flow cytometry and on autopsied human MS and non-MS tissues by immunohistochemistry.

15. SUBJECT TERMS
Adiponectin; Multiple Sclerosis; Experimental autoimmune encephalomyelitis; Blood brain barrier.

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1. INTRODUCTION

Multiple sclerosis (MS) is a presumed autoimmune CNS disorder directed against components of central nervous system (CNS) myelin affecting about 2.1 million world-wide. Current treatments are only partially effective in controlling disease activity and no drugs are available that prevent or slow MS progressive forms. Risk of MS has been associated with several environmental factors, including diet. The complex interactions between nutrition, metabolism and immune responses are just beginning to be elucidated. In this regard, MS has been studied very little. Several recent studies showed that obesity during childhood/young adulthood confers increased risk of developing MS. Obesity in the past 20 years has become an epidemic in western counties. New evidence suggests a link between obesity and several autoimmune diseases (Procaccini, Carbone et al. 2011). Obesity is characterized by a low-grade chronic inflammatory state accompanied by cytokine release that can affect immune responses (Shoelson, Herrero et al. 2007). Indeed, white adipose tissue, is an active source of cytokines, known as adipokines that regulate not only metabolic pathways, but also immune and inflammatory responses (Fantuzzi 2005, Shoelson, Herrero et al. 2007). One such adipokine is adiponectin, a protein composed of a collagenous domain and a globular domain. Adiponectin exists in the circulation in its full- length form, a functional proteolytic fragment (its globular domain) and in oligomeric forms. Adiponectin is an insulin-sensitizing molecule, but has multiple other anti-inflammatory (Yokota, Oritani et al. 2000), anti-atherogenic and vascular protective functions (Parker-Duffen, Nakamura et al. 2013) . Among its activities, adiponectin reduces the production and activities of TNFa and IL-6, down-regulates adhesion molecule expression on vascular endothelium, (Ouedraogo, Gong et al. 2007) and polarizes macrophages toward an anti-inflammatory phenotype (Ohashi, Parker et al.). Adiponectin effects are mediated through specific cell surface receptors, adiponectin receptor R1 and R2 (AdipoR1and AdipoR2), via signaling pathways that are starting to be elucidated (Mao, Kikani et al. 2006, Holland, Miller et al. 2011). AdipoR1 and R2 are expressed on various cell types including muscle, liver, endothelial cells and, as we have shown, on murine lymphocytes (Piccio, Cantoni et al. 2013). (Piccio, Cantoni et al. 2013). Other adiponectin receptors have been described (Hug, Wang et al. 2004, Takemura, Ouchi et al. 2007), and adiponectin may act in a non-receptor mediated manner via low-affinity macromolecular interactions (Okamoto, Arita et al. 2000). Activation of AMP-activated protein kinase (AMPK) has been reported downstream of AdipoRs (Mao, Kikani et al. 2006). Recently, it has been shown that adiponectin potently stimulates ceramidase activity associated with AdipoRs, and enhances ceramide catabolism and formation of it metabolite sphingosine-1-phosphate (S1P)-independently of AMPK (Holland, Miller et al. 2011). Circulating adiponectin levels display a strong inverse correlation with body mass index (BMI) and its levels are reduced in serum of overweight subjects and increased with calorie restriction (CR) (Fantuzzi 2005). The possible role of adiponectin in modulating inflammation during the MS mouse model, experimental autoimmune encephalomyelitis (EAE), was suggested by our previous studies. We have shown that CR ameliorates EAE in different mouse strains and that this was associated with altered adipokine serum levels favoring an overall anti-inflammatory milieu. Specifically we observed a decrease of leptin, a pro-inflammatory adipokine, and a significant increase in adiponectin (Piccio, Stark et al. 2008). These studies prompted us to further investigate the role of adiponectin signaling in EAE. Like MS, EAE is characterized by CNS inflammation, demyelination, and axonal damage. EAE was instrumental in the development of several therapies for MS. EAE can be induced in susceptible species by immunization with myelin antigens. Its pathogenesis consists of an initial T cell priming against myelin antigens in secondary lymphoid organs (induction phase) followed by migration across the blood brain barrier (BBB) of auto-reactive T cells and other immune cells into the CNS (effector phase). Leukocyte migration through the BBB is a critical event in EAE as well as in MS (Miller, Khan et al. 2003). The BBB strictly limits the inflow of solutes and cells from the

blood to the CNS and it is formed by endothelial cells brought together by tight junctions formed by claudins and occludins and tight liner sheets of pericytes and astrocytic endfeet (Arima, Kamimura et al.).

2. KEYWORDS

Adiponectin; Multiple Sclerosis; Experimental autoimmune encephalomyelitis; Blood brain barrier.

3. ACCOMPLISHMENTS

The accomplishments for each of the original Aims of this project is outlined below.

AIM 1.

1) Previous work from our laboratory demonstrated that T lymphocytes isolated from adiponectin knock-out (ADP KO) mice immunized to induce the MS animal model, experimental autoimmune encephalomyelitis (EAE), are more activated in vitro against the myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide, the antigen used for immunization. We also demonstrated that T lymphocytes express adiponectin receptor 1 and 2 (AdipoR1 and R2), which would suggest a direct effect of adiponectin on these cells. To better characterize the actions of adiponectin on lymphocytes, we have performed some in vitro experiments. C57BL6 mice were immunized with MOG_{35-55} and lymph node cells draining the site of immunization were isolated on day 9 post-immunization and cultured in vitro in the presence of MOG_{35-55} and MOG_{35-55} plus adiponectin (**Figure 1**). Interestingly, adiponectin induced lymphocyte proliferation in vitro in the absence of MOG_{35-55} in the culture media. This finding was consistently observed in three different experiments. This was unexpected given the fact that lack of adiponectin in ADP KO lymphocytes was associated with higher proliferation. We will further study the mechanisms leading to these phenomena in the next quarter. Specifically, we will address the specificity of this effect by silencing expression of adiponectin receptors on murine T cells.

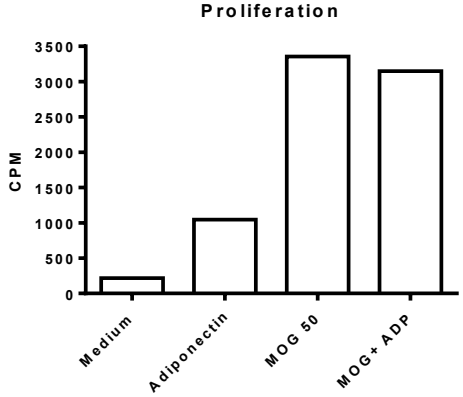

Figure 1. Lymph node cells were isolated from draining lymph nodes of MOG_{35-55} immunized C57BL/6 mice. Cells were restimulated in vitro with MOG_{35-55} in the presence or not of adiponectin. Proliferation was assessed by incorporatyion of ^{3}H – Thymidine. CPM: counts per minute.

2) We have been working on optimizing the procedure to silence expression of AdipoR 1 and R2 in murine T cells in vitro. We have used siRNA for AdipoR1 and R2 commercially available from Thermo Fisher Scientific. Up to now we have not been able to successfully transfect primary murine lymphocyte cultures with siRNA constructs. Optimization of this procedure is one of our goals for the next quarter. This is going to be critical to accomplish what proposed in Aim 1A. Alternatively, we are obtaining adiponectin receptor deficient mice (available from Jackson) that can be used as an alternative to perform the experiments proposed in Aim 1.

3) To study the signaling events activated by adiponectin in murine lymphocytes, we have performed preliminary analysis by mass spectrometry on lymph node cells isolated from ADP KO and wild type (WT) mice. Previous reports showed that adiponectin potently stimulates ceramidase activity associated with AdipoRs and enhances ceramide catabolism with formation of its metabolite sphingosine-1-phosphate. We evaluated ceramide, sphingosine and sphingosine-1-phosphate (S1P) by mass-spectrometry in lipids extracted from lymph node cells isolated from MOG_{35-55} immunized ADP KO and WT mice and stimulated in vitro. We were able to detect levels of ceramide, but no differences were noted among the various groups. We were not able to detect sphingosine and sphingosine-1-phosphate by this method. Further studies will be carried out in lymphocyte treated with adiponectin as planned in Aim1B. We will work with the facility core to optimize the detection of the metabolite we are interested in and we will study the direct effects of adiponectin on cermidase levels and AMP kinase in murine $CD4^+$ T cells.

AIM 2.

1) We have performed several experiments using the blood brain barrier (BBB) model in vitro in collaboration with Dr. Robyn Klein. We tested the direct effects on the in vitro BBB of full-length and globular adiponectin. The BBB model was generated using immortalized human cerebral microvascular endothelial cells (HCMEC) co-cultured with primary human astrocytes in a transwell system. The *in vitro* barrier integrity was assessed by measurement of transendothelial electrical resistance (TEER) or the diffusion of fluorescently labeled solutes (fluorescein-dextran of various molecular weights). **Figure 2** shows one representative experiment in which treatment with full length or globular adiponectin significantly increases TEER and decrease the diffusivity of fluorescein-dextran compared to treatment with vehicle (PBS). Treatment of the BBB with IFNγ was also included as positive control. In separate experiments we have shown that this occurs in a dose-dependent fashion (data not shown). These findings suggest that both forms of adiponectin in vitro decreases BBB permeability. We have also evaluated by immunofluorescence staining the effects of adiponectin on endothelial expression of the adhesion molecule VCAM1 and of VE-cadherin, which is important in the endothelial intercellular junction. No significant effects were observed on level of expression of VCAM and VE-cadherin on endothelial cells by treatment with full-length or globular adiponectin (**Figure 3**). These results need to be confirmed. We will also confirm the effects on the expression on endothelial cells of other adhesion molecules including ICAM1. In future studies we are going to test if the effects of adiponectin on TEER and endothelial permeability are mediated by AdipoR1 and R2. In order to do this we will perform experiments similar to what shown in Figure 2 before and after treatment with antibodies anti-AdipoR1 and R2.

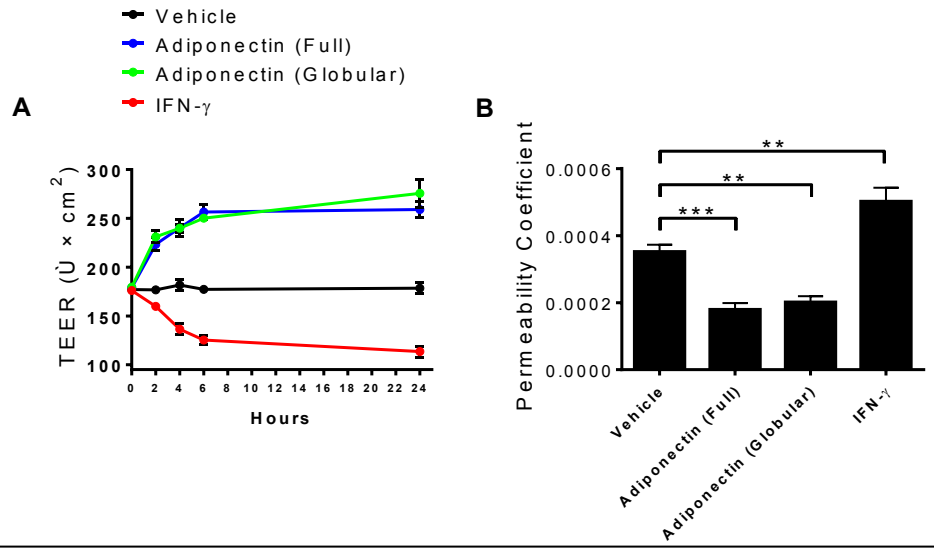

Figure 2. Treatment with full-length or globular adiponectin ***increased*** endothelial resistance (left panel) and ***decreased*** permeability (right panel) of a human BBB model compared to vehicle. IFN-gamma was a positive control in the assay. **P<0.001. ***P<0.005

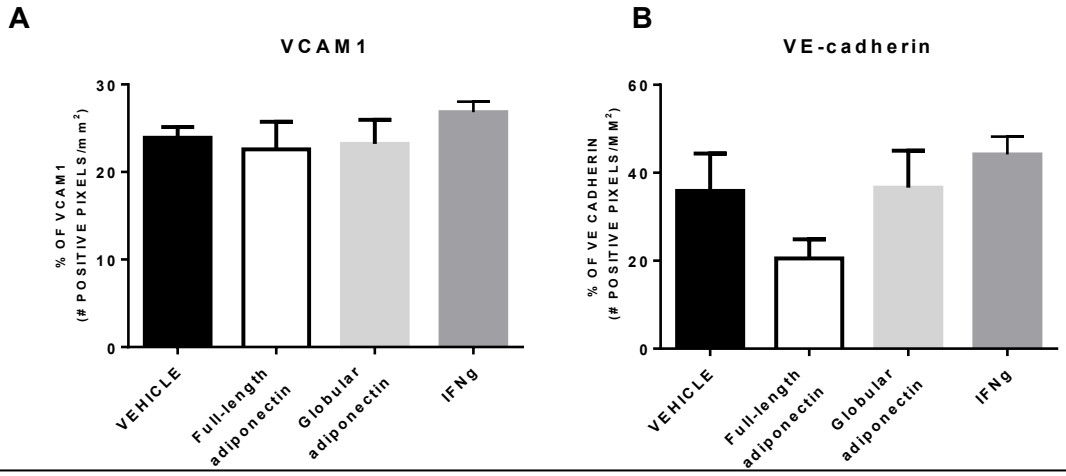

Figure 3. Expression of VCAM1 and VE-cadherin on endothelial cells in the in vitro BBB model in the conditions considered in Figure 2. VCAM1 and VE-cadherin expression was evaluated by immunofluorescence staining and quantified.

2) We evaluated expression of adiponectin receptor 1 and 2 (AdipoR1 and R2) on HCMEC and primary human astrocytes, important components of the BBB *in vitro* and *in vivo*. We have demonstrated the expression of AdipoR2 on the cell surface of the astrocytes (10% of the cells) and on small percentages of endothelial cells (0.8%). AdipoR2 was expressed intracellularly on more than 90% of the astrocytes and endothelial cells (**Figure 4**). Expression of AdipoR2 was also confirmed by immunohistochemistry (IHC) on astrocytes and endothelial cells. AdipoR1 expression was neither detected on astrocytes nor on endothelial cells by flow cytometry and immunohistochemistry (IHC).

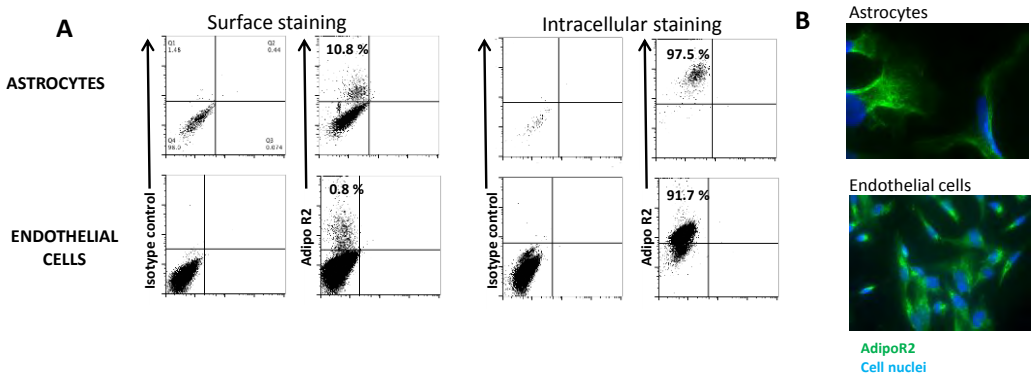

Figure 4. Adiponectin receptor 2 is expressed by human astrocytes and endothelial cells. Expression of AdipoR2 was evaluated by flow cytometry (**A**) and by IHC (**B**) on HCMEC and astrocytes. Surface staining showed AdipoR2 expression on a small proportion of astrocytes and endothelial cells, while intracellular staining showed that the majority of both cell types express the receptor.

3) We have optimized the procedure to measure BBB permeability in vivo after systemic injection of fluorescein sodium salt (**Figure 5**). Fluorescein was injected ip in C57BL/6 mice (one naïve, one with EAE and one treated with LPS). Forty-five minutes after fluorescein injection we have collected a blood sample followed by perfusion and harvesting of the brain and spinal cord. Fluorescein content of the central nervous system (CNS) relative to blood was measured for each individual mouse (permeability was assessed as the ratio between fluorescein detected in the brain and in the serum, called permeability coefficient). BBB permeability was increased in mice during EAE and after treatment with LPS, as expected. These pilot experiments demonstrated that we can detect changes in BBB permeability in vivo. In the next year we will study the effects of adiponectin in vivo in naïve mice and during EAE as outlined in Aim 2B of the original application.

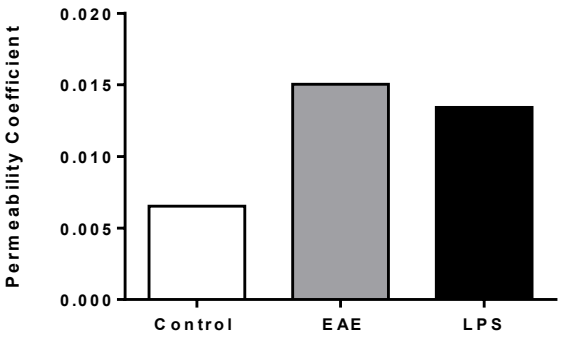

Figure 5. Measurement of BBB permeability in vivo. Fluorescein was injected in C57BL/6 mice (one naïve, one with EAE and one treated with LPS). 24h after fluorescein injection the brain was harvested and permeability was measured as the ratio between fluorescein detected in the brain and in the serum (permeability coefficient).

4) We have obtained from the laboratory of the collaborator Prof. Lily Dong detailed protocols to produce recombinant full-length and globular adiponectin. We have encountered some technical issues in producing recombinant proteins. In the next quarter we will optimize this procedure in order to successfully produce recombinant proteins that will be used in vivo and

in vitro. The experiments shown in the progress report were performed using adiponectin commercially available.

AIM 3

We have obtained official approvals to perform the human studies planned in Aim 3 of the application (use of human samples obtained ex vivo and autopsied tissues). We have now started staining peripheral blood mononuclear cells (PBMC) with anti-AdipoR1 and R2 antibodies to characterize by flow-cytometry adiponectin receptor expression in MS and healthy control subjects. The antibodies that we are currently using for the staining are commercially available, but they have not been widely used. **Figure 6** is showing staining on PBMC from a control subject in which expression of AdipoR2 has been detected on the surface and intracellular on subsets of T cells and monocytes. We will continue to systematically characterize AdipoR1 and R2 expression.

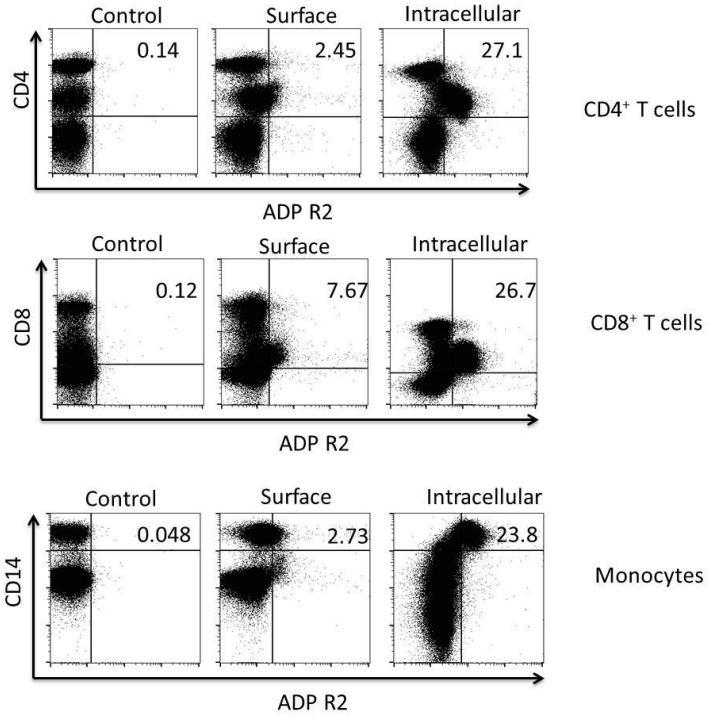

Figure 6. Flow cytometry staining of PBMC from a control subject. AdipoR2 expression has been studied on $CD4^+$, $CD8^+$ T cells and monocytes. The isotype control for AdipoR2 is shown in the first column. Surface and intracellular staining were performed. Expression of AdipoR2 has been detected on the surface of a small subset of cells, and it is also expressed at higher level intracellularly.

In addition we have started to characterize tissue blocks from human autopsied tissues that will be used in the next quarter to study AdipoR1 and R2 expression in the CNS in MS and control subjects. Tissues were stained to detect the presence of inflammatory infiltrates, myelin and myelin-laden macrophages. Based on these parameters, MS lesion were classified as active lesion, chronic inactive or active. **Table 1** summarizes the tissues and lesion types that have been characterized up to date. These will be used in the next quarter for the analyses proposed in Aim 3.

Table 1. List of autopsied tissues to be used in Aim 3.

Patient	Age	Gender	Diagnosis	Lesion Type
MS1	50	Female	SPMS	A,CI,NAWM
MS2	95	Female	SPMS	CI,NAWM
MS3	54	Female	RRMS	CI, NAWM
MS4	45	Female	SPMS	CI,NAWM
MS5	46	Female	SPMS	CI,NAWM
MS6	70	Male	SPMS	CI,NAWM
MS7	41	Female	RRMS	CI,NAWM
MS8	60	Male	SPMS	CI,NAWM
MS9	45	Female	PPMS	A,CA,CI,NAWM
C1	41	Male	---	NAWM
C2	69	Female	---	NAWM
C3	81	Male	---	NAWM

A=active lesion, CA=chronic active lesion, CI=chronic inactive lesion, NAWM= non-affected white matter. SPMS=secondary progressive MS, RRMS=relapsing-remitting MS, PPMA=primary progressive MS.

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4. IMPACT

The overall impact of this application cannot be determined yet, because this is still an ongoing project. We think that elucidating a potential role of adiponectin on the blood brain barrier will have a potential important impact on MS. Adiponectin levels in the circulation can be modulated by diet and by pharmacological intervention. This will open new avenues of therapeutic intervention for MS.

5. CHANGES/PROBLEM

Nothing to report.

6. PRODUCTS

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

No change.

8. SPECIAL REPORTING REQUIRMENTS

Not applicable.

9. APPENDICES

Nothing to append.